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Recycling pathways of glucosylceramide in BHK cells: distinct involvement of early and late endosomes

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Summary

Recycling pathways of the sphingolipid glucosylceramide were studied by employing a fluorescent analog of glucosylceramide, 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoylglucosylsphingosine (C₆-NBD-glucosylceramide). Direct recycling of the glycolipid from early endosomes to the plasma membrane occurs, as could be shown after treating the cells with the microtubule-disrupting agent nocodazole, which causes inhibition of the glycolipid's trafficking from peripheral early endosomes to centrally located late endosomes.

When the microtubuli are intact, at least part of the glucosylceramide is transported from early to late endosomes together with ricin. Interestingly, also *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE), a membrane marker of the fluid-phase endocytic pathway, is transported to this endosomal compartment. However, in contrast to both ricin and *N*-Rh-PE, the glucosylceramide can escape

from this organelle and recycle to the plasma membrane. Monensin and brefeldin A have little effect on this recycling pathway, which would exclude extensive involvement of early Golgi compartments in recycling. Hence, the small fraction of the glycolipid that colocalizes with transferrin (Tf) in the Golgi area might directly recycle via the *trans*-Golgi network. When the intracellular pH was lowered to 5.5, recycling was drastically reduced, in accordance with the impeding effect of low intracellular pH on *vesicular transport* during endocytosis and in the biosynthetic pathway.

Our results thus demonstrate the existence of at least two recycling pathways for glucosylceramide and indicate the relevance of *early endosomes* in recycling of both proteins and lipids.

Key words: early endosome, fluorescence microscopy, glycolipid, Golgi, recycling.

Introduction

Recycling is a well-known feature of intracellular protein trafficking, occurring during endocytosis (Brown et al. 1983; Podbilewicz and Mellman, 1990; Steinman et al. 1983). Recycling is defined as the inter-organelle movement of a molecule followed by its return to the original site, without intervening metabolic processing. More specifically, it occurs for instance in the endocytic pathway of a receptor-ligand complex, involving internalization and return of the *unmodified* and *intact* receptor to the plasma membrane.

The potential importance of this process in the concomitant intracellular flow of lipids is now also emerging. Several sphingolipids have been shown to reappear at the plasma membrane by a recycling mechanism, after exogenous membrane insertion and subsequent internalization by endocytosis (Kok et al. 1989; Koval and Pagano, 1989, 1990). In this respect glycolipids may be of special interest, since most species belonging to this class of lipids are

thought to reside mainly in the outer leaflet of the plasma membrane, in accordance with their functions in cell surface recognition phenomena (Curatalo, 1987; Hakomori, 1981; Hoekstra and Düzgünes, 1989). Recently we have presented direct evidence that the neutral glycolipid glucosylceramide is sorted during inbound cellular trafficking, thus avoiding degradation in the lysosomal compartment (Kok et al. 1991). By inference, similar conclusions can be drawn from work described by others (Trinchera et al. 1990; Usuki et al. 1988), in which the same class of lipids was studied.

Internalization and recycling pathways can be characterized by making use of ligand-receptor complexes with a well-known intracellular cycle. The iron transporter transferrin (Tf) is rapidly internalized to early endosomes, an organelle which appears to play a pivotal role in intracellular sorting (Stein and Sussman, 1986; Stoorvogel et al. 1987). After iron-discharge the apo-transferrin, still bound to its receptor, recycles back to the plasma membrane from these early endosomes. A minor fraction recycles via the

Golgi region (Stein and Sussman, 1986). Several other ligands, like ricin, move further down the endocytic pathway towards the lysosomal system.

In the present study it is shown that a fluorescent analog of the glycolipid glucosylceramide can - like transferrin - directly recycle from early endosomes to the plasma membrane after initial uptake into the cell along the pathway of receptor-mediated endocytosis. From these early endosomes a fraction of the glycolipid - but not transferrin - can be transported to late endosomes along an identical route to that for ricin, a marker for the endocytic pathway towards the endo-lysosomal system. However, in contrast to ricin, glucosylceramide is only transiently present in this organelle as it can escape from this compartment and subsequently return to the plasma membrane.

Materials and methods

Materials

1- β -D-glucosylsphingosine, D-sphingosine, iron-free human transferrin (Tf), monensin, nocodazole, brefeldin A (BFA) and cytochalasin D were purchased from Sigma. 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoic acid (C₆-NBD), lissamine rhodamine B sulfonyl chloride (LR), 10% (w/w) on celite, N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (N-Rh-PE), propidium iodide and bodipy-phalloidin were from Molecular Probes. Anti- α -tubulin antibody was from Amersham. Anti-mouse antibodies (FITC-conjugated) were from Dakopatts (Denmark). Ricin RCA60 was a gift from Dr. E. Nicolas, Université René Descartes, Department of Biochemistry, Paris.

Synthesis of C₆-NBD-labeled sphingolipids

C₆-NBD-glucosylceramide and C₆-NBD-ceramide were synthesized from C₆-NBD and 1- β -D-glucosylsphingosine and D-sphingosine, respectively, as described (Kishimoto, 1975). The C₆-NBD lipids were quantitated spectrophotometrically in a fluorometer (Perkin-Elmer MPF-43) with an excitation wavelength of 465 nm and an emission wavelength of 530 nm, by reference to a known amount of C₆-NBD-phosphatidylcholine.

Protein labeling

Saturation of Tf with iron was carried out by the procedure of Van Renswoude et al. (1982). Diferric Tf and ricin were conjugated with LR as described elsewhere (Kok et al. 1989).

Cell culture and membrane insertion of fluorescent lipids

Monolayer cultures of baby hamster kidney cells (BHK-21) were grown in Glasgow minimum essential medium, supplemented with 5% (v/v) fetal calf serum and 10% (v/v) tryptose phosphate broth in a water-saturated atmosphere of 5% CO₂ (95% air). Experiments were carried out 48-72 h after passage. C₆-NBD-lipid insertion was carried out at 2°C. Prior to labeling, the cells were cooled to 2°C (30 min). Two procedures were employed to insert the fluorescent lipid analog into the plasma membrane of the cells. (i) The cells were incubated with donor membranes, consisting of C₆-NBD-lipid-containing liposomes. To this end, small unilamellar vesicles (190 nmol of dioleoylphosphatidylcholine and 10 nmol of C₆-NBD-lipid in 1 ml of Hanks' solution) were freshly prepared for each experiment by probe sonication for 10-15 min on ice and under nitrogen. (ii) Alternatively, an appropriate amount of C₆-NBD-lipid, stored in chloroform/methanol (2:1, v/v), was dried under nitrogen and subsequently solubilized in absolute ethanol. A sample of the ethanolic solution (0.5%, final concen-

tration) was injected into Hanks' solution (pH 7.4) under vigorous vortex mixing. This solution was then added to the cells. The latter method was also used for the insertion of N-Rh-PE (Kok et al. 1990).

Lipid and protein internalization

Labeling of the cells with LR-Tf (0.2 mg/ml) or LR-ricin (15 µg/ml) was carried out for 30 min at 2°C, whereafter the unbound Tf or ricin was removed by washing the cells with ice-cold Hanks' solution. To initiate the internalization of the membrane-inserted lipid and the membrane-bound protein ligand, the cells were warmed to 37°C by adding warm Hanks' solution. At various times, the buffer was removed and replaced by ice-cold (2°C) Hanks' solution.

Back-exchange of membrane-inserted lipid analogs

A back-exchange procedure was carried out by incubating the cells for 30 min at 2°C with 5% (w/v) bovine serum albumin (BSA) in Hanks' solution, followed by extensive washings with Hanks' solution and another incubation with 5% BSA in Hanks' solution (30 min at 2°C). Fluorescence was measured after lipid extraction of the BSA solution and the cells, to determine the back-exchanged fraction, and to calculate the percentage of internalization or recycling.

Lipid extraction, analysis and quantification

Lipids were extracted by the procedure of Bligh and Dyer (1959) and analyzed by thin-layer chromatography (TLC) on silica gel 60 HPTLC plates (Merck), using CHCl₃/CH₃OH/20% (w/v)-NH₄OH (70:30:5, by vol.) as the running solvent system. For quantification, individual spots were scraped off the plates and after addition of 3 ml of a 1% (v/v) Triton X-100 solution, shaken vigorously for 1 h at 37°C. After spinning down the silica particles, the supernatant was measured by reference to a standard curve, as described above. Corrections were made for differences in efficiency of removal of different lipids from the silica.

Recycling

Microscopic visualization of lipid recycling and chemical analysis of internalized and recycled lipids were done as follows. The cells were labeled at 2°C with C₆-NBD-glycolipid. Internalization was then triggered by warming the cells to 37°C. After 30 min at 37°C, the cells were cooled and a back-exchange procedure was carried out at 2°C, as described above, to remove all residual plasma membrane-incorporated NBD-lipid. After this back-exchange, the cells were again warmed to 37°C for various times, either in Hanks' buffer or in the same buffer supplemented with 5% BSA (w/v). Finally, after cooling, a second back-exchange was carried out, if appropriate. For analysis of the chemical nature of the cell-associated and back-exchanged fluorescent molecules, each relevant fraction was subjected to a lipid extraction.

The effects of monensin, BFA, metabolic inhibitors and cytosol acidification on recycling were studied as follows. In the case of BFA treatment, the cells were preincubated with BFA (2 µg/ml) in Hanks' solution for 30 min at 37°C and all subsequent incubations at 37°C were carried out in the presence of BFA. Cells were labeled with 4 µM C₆-NBD-glucosylceramide at 2°C. The NBD-glycolipid was then allowed to internalize, as accomplished by raising the temperature to 37°C. After an appropriate time interval, the remaining pool of plasma membrane associated lipid was removed by a back-exchange procedure. Subsequently, the cells were incubated at 2°C for 30 min with 10 µM monensin, 5 mM sodium azide/50 mM 2-deoxyglucose or, to acidify the cytosol, in a 5 µM nigericin-containing isotonic KCl buffer solution (140 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 0.5 mM KH₂PO₄, 20 mM Hepes, pH 5.5). (Owing to the ability of nigericin to exchange K⁺ for

H⁺, the pH of the cytosol will equilibrate with that of the surrounding medium when the concentration of K⁺ is the same at either side of the membrane (Frelin et al. 1988; Grinstein et al. 1984).) Thereafter the cells were warmed to 37°C for 45 min in the presence of the drugs. Finally, a second back-exchange procedure was performed to determine the amount of recycled lipid. The effect of monensin or BFA on transport from the Golgi apparatus to the plasma membrane was established as follows: if appropriate, the cells were pre-incubated with BFA (2 µg/ml) for 30 min at 37°C. The cells were labeled at 2°C for 30 min with 4 µM C₆-NBD-ceramide. This was followed by an incubation with 10 µM monensin at 2°C for 30 min if appropriate. Subsequently the cells were warmed to 37°C for 45 min in the presence of either drug.

In the case of both glucosylceramide recycling and transport of newly synthesized glucosylceramide the transport to the plasma membrane in drug-treated cells relative to that in control cells is expressed using the equation:

$$R = \frac{PM_T / (PM_T + I_T)}{PM_C / (PM_C + I_C)}$$

(PM, plasma membrane glucosylceramide pool; I, intracellular glucosylceramide pool; T, treated cells; C, control cells), yielding a value of zero in the case of complete inhibition by the drug and unity if the drug has no effect.

Cytoskeletal disruption

To study the involvement of the cytoskeleton in the process of internalization and recycling of NBD-glucosylceramide, cells were preincubated with either nocodazole (10 µg/ml) for 90 min at 37°C or cytochalasin D (10 µg/ml) for 15 min at 37°C to disrupt microtubuli or actin filaments, respectively. All subsequent incubations were carried out in the presence of either drug. To determine the kinetics of breakdown and recovery (after removal of the drug by extensive washing) of these cytoskeletal elements, indirect immunofluorescence microscopy was employed. The cells were fixed for 10 min in 4% formaldehyde in a cytoskeleton-stabilizing buffer (100 mM Pipes, 5 mM EGTA, 2 mM MgCl₂, pH 6.8) containing 0.1% Triton X-100. After washing the specimen with PBS, the cells were post-fixed for 5 min in -20°C methanol or ethanol for tubulin or actin labeling, respectively. After repeated PBS washings the cells were incubated with 10% fetal calf serum to saturate non-specific protein binding sites. In the case of tubulin staining, the cells were incubated with anti- α -tubulin antibodies at room temperature for 30 min and, after extensive washing with PBS, with FITC-conjugated anti-mouse antibodies for 30 min. For actin staining, bodipy-conjugated phalloidin was used.

Microscopy

Cells were grown on glass coverslips in 35-mm Petri dishes. Prior to experiments, the cells were cooled (30 min) and washed several times with ice-cold Hanks' solution. For colabeling studies, the cells were first labeled with the C₆-NBD-lipid for 30 min at 2°C followed by washing with Hanks' solution and subsequent labeling with LR-Tf (0.2 mg/ml), LR-ricin (15 µg/ml) or N-RhPE (1 µM). Internalization was initiated by adding warm (37°C) Hanks' solution, and after various times the trafficking processes were stopped by replacing warm buffer with ice-cold buffer. Thereafter a back-exchange was carried out at 2°C, followed, in the case of ricin labeling, by a 0.1 M lactose wash (30 min at 2°C) to remove cell-surface-bound ricin. To examine the effect of BFA on glucosylceramide trafficking, cells were preincubated with BFA (2 µg/ml) for 30 min at 37°C, followed by labeling with C₆-NBD-glucosylceramide at 2°C and subsequent internalization for 30 min at 37°C in the presence of the drug. The BFA effect on the Golgi apparatus was assessed by first labeling the Golgi

apparatus with C₆-NBD-ceramide during 30 min at 37°C, followed by another 30 min incubation at 37°C in the presence of BFA. Cell viability was checked in all microscopic experiments by addition of propidium iodide (10 µg/ml) to the incubation medium. This dye is excluded from viable cells but stains nuclei of disrupted cells.

In some experiments the cells were fixed for 30 min at room temperature with 2% (v/v) formaldehyde in buffer containing 20 mM sodium phosphate (pH 7.4), 100 mM lysine, 60 mM sucrose and 100 mM sodium periodate, post-fixed sequentially with 4% formaldehyde and with 6% formaldehyde, both in the same buffer, for 5 and 10 min, respectively.

Fluorescence microscopy was performed with a Leitz Orthoplan or a Zeiss Axiovert 405 M microscope, using filter sets, as described elsewhere (Kok et al. 1990). Photomicrographs were taken at 10–30 s exposition times using Kodak T-max P3200 film that was processed at 3200 up to 12,800 ASA.

Miscellaneous procedures

Protein was determined by the Lowry procedure, modified as described by Peterson (1977). Bovine serum albumin was used as a standard. Phospholipid phosphorus was determined after perchloric acid destruction according to Böttcher et al. (1961).

Results and discussion

Internalization and recycling kinetics

C₆-NBD-glucosylceramide can be readily inserted into the outer leaflet of the plasma membrane of BHK cells at 2°C. When the cells are subsequently warmed to 37°C, the glycolipid is internalized via the pathway of receptor-mediated endocytosis (Kok et al. 1989). Fig. 1 shows the kinetics by which the lipid is removed from the plasma membrane and gains intracellular access. Initial internalization occurs at a relatively rapid rate as after only 2 min, ~25% of the maximal internalized amount (under steady-state conditions) is found intracellularly. At steady-state conditions ~20% of the total cell-associated lipid fraction resides inside the cell, which implies that a relatively high percentage of the total lipid pool remains associated with the plasma membrane, either because of its persistent presence in the plasma membrane as such or by recycling to the plasma membrane after initial uptake into the cell.

Recycling of internalized glycolipid to the plasma membrane, i.e. the return of *intact* molecules that have not been modified metabolically, was measured next. The cells were labeled with the glycolipid and internalization was allowed to proceed for 30 min. After 30 min at 37°C a steady state is reached in the distribution of the intracellular labeling pattern (Kok et al. 1989), indicating that all possible organelles to be reached by the glycolipid are labeled at that time point. Subsequently a back-exchange procedure is carried out to remove the residual pool of plasma membrane-associated NBD-glycolipid. The overall kinetics of recycling from intracellular organelles can now be established by determining the time of reappearance of intact C₆-NBD-glucosylceramide at the plasma membrane occurring upon further incubating the cells at 37°C for various time intervals (Fig. 2a). The early kinetics of recycling are extremely fast as approx. 20% of the total fraction involved in recycling during a period of 60 min can be recovered

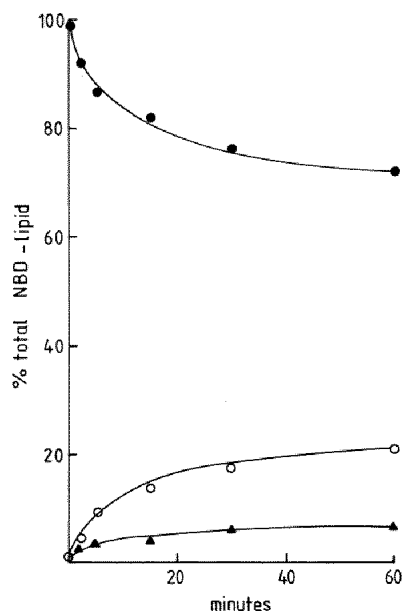


Fig. 1. Kinetics of internalization of C₆-NBD-glucosylceramide in BHK cells. Cells were labeled with 4 μ M C₆-NBD-glucosylceramide and allowed to internalize the lipid at 37°C for various time intervals. After a subsequent back-exchange procedure NBD-lipid was quantified in the cell fraction (○—○, intracellular), the back-exchange fraction (●—●, plasma membrane) and the 37°C incubation medium (▲—▲, expelled in medium), as described in Materials and methods, and expressed as the percentage of total NBD-lipid in all three fractions. The 100% value corresponds to approximately 55 pmol/10⁶ cells. Data points are the means of duplicate measurements.

during the first minute. After about 1 h, recycling seems to level off, yielding a ratio of plasma membrane fraction to internal fraction which is comparable to that reached during internalization (Fig. 1). However, once recycled to the plasma membrane, NBD-glycolipid molecules may well be subject to another internalization cycle, thus contributing to the kinetics monitored during a 60 min recycling experiment. To investigate this issue, an alternative recycling experiment was performed in which the cells were incubated in Hanks' solution containing 5% (w/v) BSA during recycling. The presence of BSA as NBD-glycolipid acceptor will ensure that any C₆-NBD-glycolipid molecule that recycles to the plasma membrane will be effectively removed prior to its ability to engage in a second round of internalization. As shown in Fig. 2b, the kinetics of recycling are indeed different under these conditions. The disappearance of the glycolipid from intracellular organelles is more extensive and after 60 min only 5% has not yet returned to the cell surface. By contrast, in the absence of BSA, approximately 30% of the lipid fraction is located intracellularly. From the kinetic curve obtained in the presence of BSA, which thus represents true recycling without interference of repeated internalization, a half-life of 12.5 min for C₆-NBD-glucosylceramide can be calculated.

From the distinction in the kinetics of recycling as revealed by a comparison of the rates in the absence and presence of BSA (Fig. 2b), it is clear that after their recy-

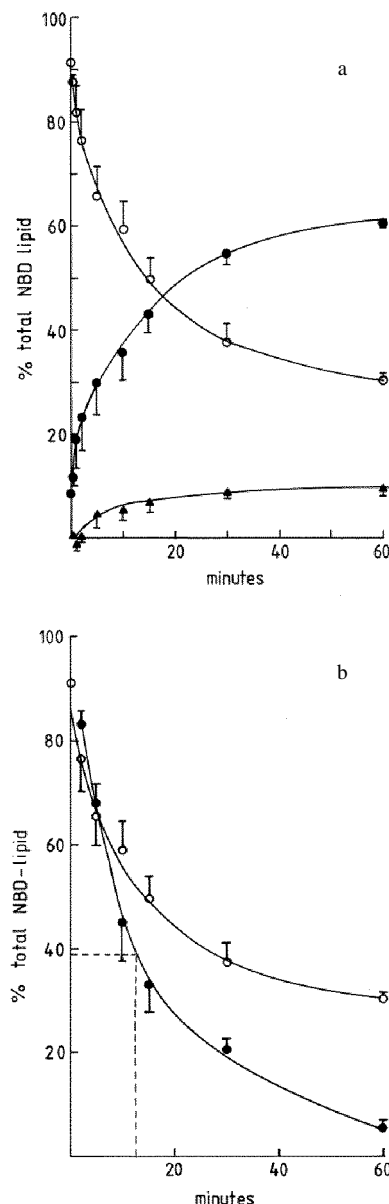


Fig. 2. Kinetics of recycling of C₆-NBD-glucosylceramide from intracellular organelles to the plasma membrane of BHK cells. Cells were labeled with 4 μ M C₆-NBD-glucosylceramide and allowed to internalize the lipid for 30 min at 37°C. Thereafter a back-exchange procedure was carried out to remove all residual plasma membrane NBD-lipid. Subsequently the cells were warmed again to 37°C for various time intervals, followed by a second back-exchange procedure. The latter removes the NBD-lipid that has recycled from intracellular organelles to the plasma membrane. NBD-lipid was quantified, as described in Materials and methods, in the cell fraction (○—○, intracellular), the second back-exchange fraction (●—●, plasma membrane: recycled fraction) and the 37°C incubation medium (▲—▲, expelled in medium), and expressed as the percentage of total NBD-lipid in all three fractions (a). The 100% value corresponds to approximately 11 pmol/10⁶ cells. In (b) the percentage of total NBD-lipid, which is present intracellularly is depicted, both in the absence (○—○) and presence (●—●) of BSA in the 37°C medium (5% w/v) as a NBD-lipid scavenger. Note that curve (○—○) is identical to curve (○—○) of (a). Data points are the means (\pm s.e.m.) of triplicate measurements.

cling to the plasma membrane, glycolipid molecules may re-enter the internalization pathway. This eventually leads to a steady state in which 70-80% of the glycolipid resides in the plasma membrane while the remaining fraction is located intracellularly. This distribution of the NBD-glycolipid is in reasonable agreement with measurements of the surface area of the plasma membrane versus the endosomal apparatus (Griffiths *et al.* 1989), indicating that under steady-state conditions the glycolipid is distributed approximately according to membrane surface area.

Consistent with previous observations (Kok *et al.* 1989), the recycled fraction consists exclusively of intact C₆-NBD-glucosylceramide, as revealed by lipid extraction and thin layer chromatography (not shown). Thus, after recycling the only lipid the plasma membrane contains is this NBD-labeled (sphingo)lipid. In this context, it should be noted that metabolic experiments employing C₆-NBD-ceramide as a precursor for sphingolipid synthesis have shown that both C₆-NBD-glucosylceramide and C₆-NBD-sphingomyelin are formed and transported to the plasma membrane, in a ratio of glucosylceramide to sphingomyelin of 0.85 (unpublished results). Thus substantial degradation of glucosylceramide to ceramide should have resulted in a significant synthesis of sphingomyelin and its subsequent transport to the plasma membrane. Since during the time course of the recycling experiments no C₆-NBD-sphingomyelin is found, we conclude that all C₆-NBD-glucosylceramide present in the plasma membrane stems from genuine recycling of intact molecules, i.e. molecules that have not been processed metabolically. Finally, it is relevant to note that the recycling experiment (Fig. 2b) in the presence of BSA reveals that essentially the entire pool of internalized lipid can be recycled within a time interval of about one hour. Hence, recycling of glucosylceramide is not restricted to a particular fraction of the intracellular pool.

Internalization and recycling pathways: the early endosomal level

C₆-NBD-glucosylceramide is internalized along the pathway of receptor-mediated endocytosis. Two minutes after triggering internalization, the lipid analog is located in early endosomes, as inferred from its colocalization with LR-Tf: transferrin is by definition a marker for early endosomes after 2 min of uptake (Eskelinen *et al.* 1991; Chiechanover *et al.* 1983; Dautry-Varsat *et al.* 1983; Van Renswoude *et al.* 1982). When colabeling experiments were performed with LR-ricin, this marker was similarly found to colocalize with the NBD-glycolipid at the early endosomal level, 2 min after initiating internalization (cf. Fig. 4). However, the early endosomes were not labeled by N-Rh-PE (cf. Fig. 5), a membrane traffic marker of the fluid-phase endocytic pathway (Kok *et al.* 1990).

As shown in Fig. 3, trafficking from early to late endosomes can be reversibly blocked by incubating the cells with nocodazole, which disrupts the microtubular system. (To ascertain the effectiveness of disruption and its reversibility, the kinetics of breakdown and recovery were monitored microscopically by indirect immunofluorescent staining of α -tubulin; not shown.) The compartment in which C₆-NBD-glucosylceramide accumulates during a 30 min incubation at 37°C after nocodazole treatment is the

same compartment as that labeled by LR-Tf, when this ligand is allowed to internalize for only 2 min at 37°C (Fig. 3). This indicates that the block of glucosylceramide trafficking is indeed at the level of the early endosomes, which by definition are labeled by transferrin after 2 min of uptake. Indeed, in support of our claim is the observation that transfer of VSV G protein from early to late endosomes in BHK cells is also blocked in the presence of nocodazole (Gruenberg *et al.* 1989). After a 30 min accumulation of C₆-NBD-glucosylceramide in early endosomes, a back-exchange was performed to remove residual plasma membrane NBD-glycolipid. When the incubation of the cells is subsequently continued at 37°C, still in the presence of nocodazole, the glycolipid recycles from early endosomes to the plasma membrane, as shown in Fig. 3. It thus follows that microtubuli are not required for recycling of the glycolipid via early endosomes, consistent with observations on the recycling of Tf (Sakai *et al.* 1991); whereas delivery of endocytosed Tf to the cytocenter is blocked, recycling to the plasma membrane proceeds normally.

Internalization and recycling pathways: the late endosomal level

Under normal conditions, i.e. when the microtubular system is unperturbed, C₆-NBD-glucosylceramide not only recycles from early (peripheral) endosomes to the plasma membrane, but part of the lipid analog is transported further into the cell, towards the cell center. After 5 to 15 min of internalization large spherical vesicles become labeled (Kok *et al.* 1989), which are localized more centrally compared to early endosomes. In addition, some labeling can also be found in the Golgi area, a distinct juxtanuclear area in BHK cells. As shown in Fig. 4, colocalization of the glycolipid with LR-Tf is, after longer times of incubation (15 min), restricted to this Golgi area, since transferrin is not found in the large spherical vesicles. In order to identify the latter vesicles, colabeling experiments were performed with other endocytic markers. As shown in Fig. 4, colabeling with LR-ricin revealed that C₆-NBD-glucosylceramide is transported together with this protein ligand, not only through early endosomes, but also to the large spherical vesicles, where both probes colocalize up to incubation times of 30 min. Only after 60 min at 37°C, did the labeling pattern of LR-ricin deviate from that of the NBD-glycolipid. At that time LR-ricin was present in very large spherical vesicles, most likely lysosomes, since N-Rh-PE also accumulates in large spherical lysosomes after 60 min of uptake, as revealed by density gradient analyses (Kok *et al.* 1990). In this compartment C₆-NBD-glucosylceramide was never found (Fig. 4).

After 15 min of internalization, C₆-NBD-glucosylceramide was also observed to colocalize with N-Rh-PE in the large spherical vesicles, as shown in Fig. 5. Interestingly, in contrast to N-Rh-PE, C₆-NBD-glucosylceramide could be chased out of this compartment. This was accomplished by incubating the cells further at 37°C for 5 to 10 min, after first having removed the plasma membrane pool by a back-exchange (Fig. 5). Similar observations were made in the case of the colocalization studies with LR-ricin. Whereas the NBD-glycolipid could be chased from this organelle, the ricin could not (not shown). Apparently the

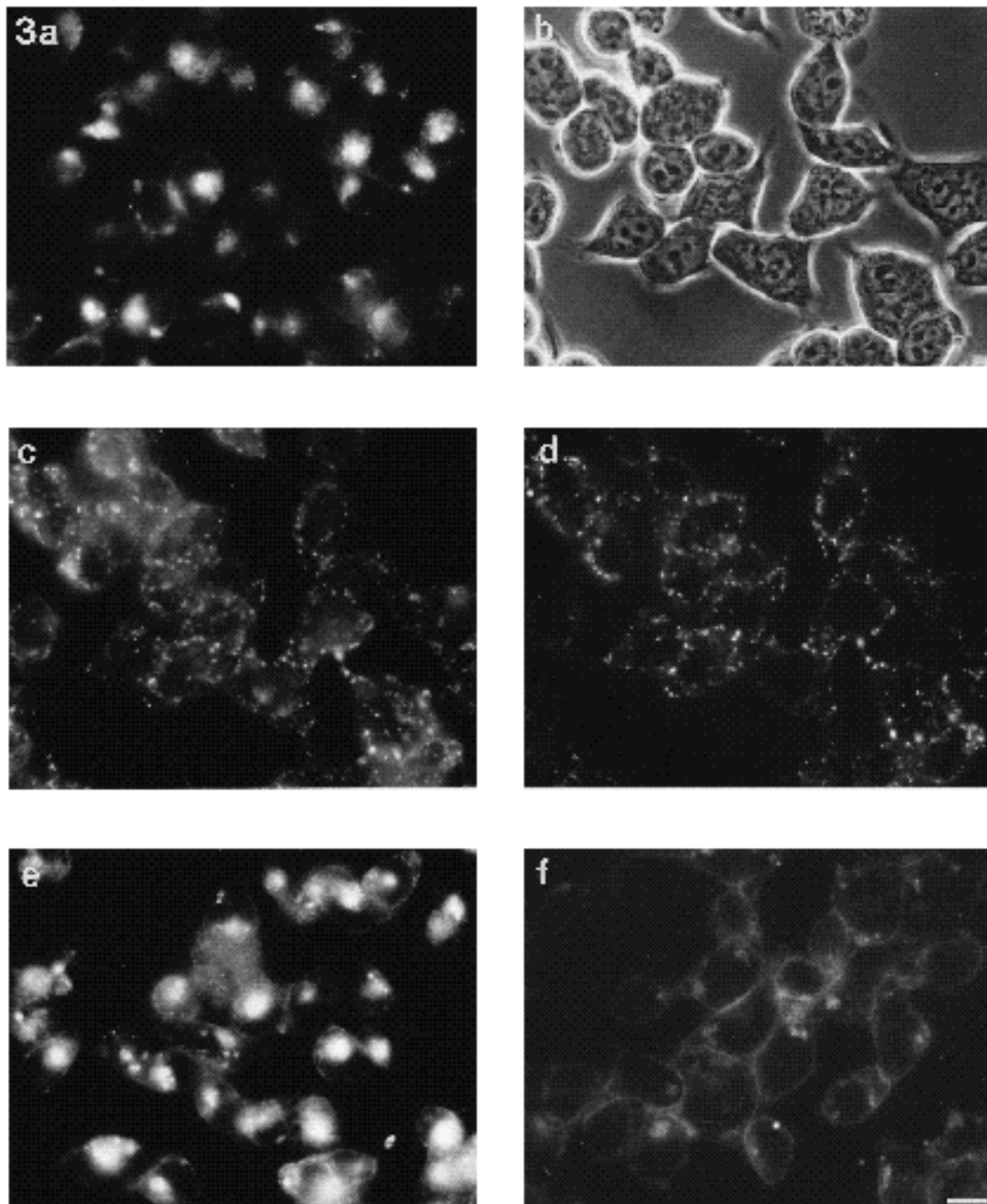


Fig. 3. Reversible inhibition of transport of C₆-NBD-glucosylceramide to late endosomes by nocodazole and direct recycling from early endosomes to the plasma membrane. In all samples the cells were pretreated as indicated and subsequently labeled with 4 μ M C₆-NBD-glucosylceramide, followed by incubation at 37°C for 30 min and finally a back-exchange procedure. (a) Control cells (i.e. not treated with nocodazole) were preincubated for 90 min in Hanks' solution. Fluorescent glucosylceramide accumulates in a central region. (c) Cells were preincubated for 90 min in Hanks' solution containing 10 μ g/ml nocodazole to disrupt microtubuli. Fluorescent glucosylceramide does not move from a peripheral location in the cell to the central region as in a. (d) The same sample as in c was labeled with LR-Tf (0.2 mg/ml), which was allowed to internalize for only 2 min at 37°C. By definition the transferrin reaches the early endosomes. Note that Tf (d) colocalizes with glucosylceramide (c) which has been internalized for 30 min. Thus glucosylceramide (c) does not move beyond the early endosomal level during prolonged incubation in nocodazole-treated cells. (b) Phase-contrast image corresponding to c and d. (e) After microtubuli disruption (nocodazole, 90 min), the cells were allowed to recover for 90 min in Hanks' solution at 37°C. Thereafter C₆-NBD-glucosylceramide internalization proceeds normally, resulting in a labeling pattern resembling that in a (control), but distinct from that in c. Thus inhibition of glucosylceramide trafficking to the central cell region is reversible. (f) After internalization in the presence of nocodazole (compare c) and back-exchange (to remove residual plasma membrane NBD-lipid) the cells were further incubated at 37°C for 45 min. The plasma membrane staining indicates that recycling has occurred from peripheral early endosomes. Bar, 10 μ m.

glycolipid can escape from this compartment and, unlike *N*-Rh-PE and ricin, does not move further to the lysosomal system but, rather, appears to be able to recycle to the plasma membrane. Thus the large spherical vesicles that contain C₆-NBD-glucosylceramide after 15 min of uptake are kinetically intermediate between early endosomes (reached after 2 min of uptake of the glycolipid) and lysosomes (never reached by the glycolipid but for instance by *N*-Rh-PE after 60 min of uptake). Therefore these vesicles can be considered to be late endosomes.

The experimental results indicate that apart from recycling via early endosomes, some of the lipid can be transported to the Golgi area, as occurs for Tf, while another fraction reaches late endosomes, following the pathway along which ricin is transferred. These events are schematically depicted in Fig. 6. It should be noted that beyond the level of early endosomes, the pathways of Tf and the glycolipid are partly separated. At the late endosomal level the receptor-mediated endocytic pathway, taken by a fraction of the NBD-glycolipid, converges with the fluid-phase endocytic pathway (Fig. 6), for which *N*-Rh-PE is used as a membrane marker (Kok et al. 1990). In this context, note that in contrast to the late endosomal level (Fig. 5 c,d), there is no colocalization of C₆-NBD-glucosylceramide and *N*-Rh-PE at the early endosomal level (Fig. 5 a,b). This indicates that a 'cross-over' occurs between these two endocytic pathways at the level of the late endosome. Apparently, in this late endosomal compartment the NBD-glycolipid is sorted (e.g. from *N*-Rh-PE), allowing it to recycle to the plasma membrane.

Involvement of the Golgi apparatus in recycling

After longer incubation times (15 min) part of the NBD-glucosylceramide colocalizes with LR-Tf in the Golgi area (see above). In order to establish whether the Golgi apparatus is involved in recycling of the glycolipid, experiments were carried out using monensin to disrupt the Golgi system and to inhibit transport to the plasma membrane. The transport to the plasma membrane of newly synthesized C₆-NBD-glucosylceramide (or sphingomyelin) from the precursor C₆-NBD-ceramide in the Golgi apparatus, is ~50% reduced in monensin-treated cells (Table 1). However, only a minor effect of monensin is seen on the recycling of intact C₆-NBD-glucosylceramide after its initial uptake by endocytosis. Furthermore, as shown in Fig. 7, the labeling pattern observed after internalization of C₆-NBD-glucosylceramide is not changed when the cells are pretreated with BFA (compare d vs. c), causing redistribution of the Golgi system into the endoplasmic reticulum (Lippincott-Schwartz et al. 1990), as demonstrated here (compare a vs. b) by the distinct difference in the distribution of the Golgi marker C₆-NBD-ceramide. Also recycling of the glycolipid was not affected, as judged by fluorescence microscopy (not shown) and based on quantifying the recycled fraction (Table 1). However, the transport of newly synthesized glucosylceramide from the Golgi to the plasma membrane is also unaffected by BFA treatment, in spite of the drastic morphological changes (Fig. 7).

In conclusion, monensin hardly affects overall recycling while BFA, which is known to cause redistribution of the *cis* to *trans* Golgi compartments into the ER (Lippincott-

Table 1. Effect of monensin or BFA on C₆-NBD-glucosylceramide recycling

	Relative transport to plasma membrane (<i>R</i>)	
	Glucosylceramide synthesized in Golgi apparatus	Glucosylceramide recycling
Control	1	1
Monensin	0.51	0.85
BFA	0.99	1.06

In the case of BFA treatment, BHK cells were preincubated with BFA (2 µg/ml) in Hanks' solution for 30 min at 37°C. All subsequent incubations were also carried out in the presence of BFA. The cells were labeled with 4 µM C₆-NBD-glucosylceramide and allowed to internalize the lipid for 30 min at 37°C, whereafter a back-exchange procedure was carried out to remove all residual plasma membrane NBD-lipid. In the case of monensin-treatment, this was followed by a preincubation with Hanks' solution containing 10 µM monensin for 30 min at 2°C. Subsequently, recycling was allowed to proceed for 45 min by incubating the cells at 37°C, in either the presence (monensin or BFA) or the absence (control) of drugs. Thereafter a final back-exchange procedure was performed to analyse the C₆-NBD-lipid present in this (recycling) fraction and the residual intracellular fraction. From these data the relative transport factor *R* was calculated, as described in Materials and methods.

In a parallel experiment the effect of monensin or BFA on transport of newly synthesized C₆-NBD-glucosylceramide from the Golgi to the plasma membrane was examined. In the case of BFA treatment, the cells were preincubated with BFA (2 µg/ml) in Hanks' solution for 30 min at 37°C. The cells were labeled with 4 µM C₆-NBD-ceramide at 2°C for 30 min. In the case of monensin-treatment this was followed by a preincubation with Hanks' solution containing 10 µM monensin for 30 min at 2°C. Subsequently, the cells were incubated at 37°C for 45 min, followed by a back-exchange procedure to separate plasma membrane and intracellular fractions. Data are the means of triplicate measurements with standard deviations <10%.

Schwartz et al. 1990), does not affect the labeling pattern obtained after C₆-NBD-glucosylceramide internalization, including the Golgi-area labeling. Therefore the *cis* to *trans* Golgi compartments do not seem to be involved in trafficking of endocytosed glucosylceramide. However, the *trans*-Golgi network (TGN), an important sorting organelle (Griffiths and Simons, 1986), is a possible candidate for involvement in recycling of glucosylceramide. Monensin, although affecting early transport steps of sphingolipids after synthesis (Table 1), may not affect cycling, occurring directly through the TGN. Golgi area labeling would still be observed in BFA-treated cells, when the TGN is not affected by this treatment. Recently, BFA was reported to affect organelles in the endocytic pathway as well (Wood et al. 1991; Lippincott-Schwartz et al. 1991; Pelham, 1991). Most notably, the drug appears to cause fusion of the TGN with early endosomes in certain cell types. Possibly, this phenomenon may either not occur in BHK cells or does not lead to drastic morphological changes, given the similar labeling patterns of glucosylceramide recycling organelles in both BFA-treated and control cells (Fig. 7). Alternatively, the Golgi area labeling may represent another type of endosomal vesicle, in addition to the early and late endosomes described above. In BHK cells, endosomal vesicles can be found very close to the TGN (Griffiths and Simons, 1986). Therefore, resolving this issue ultimately depends on the availability of methods for electron microscopic visualization of glucosylceramide in relation to established Golgi markers, including specific TGN markers.

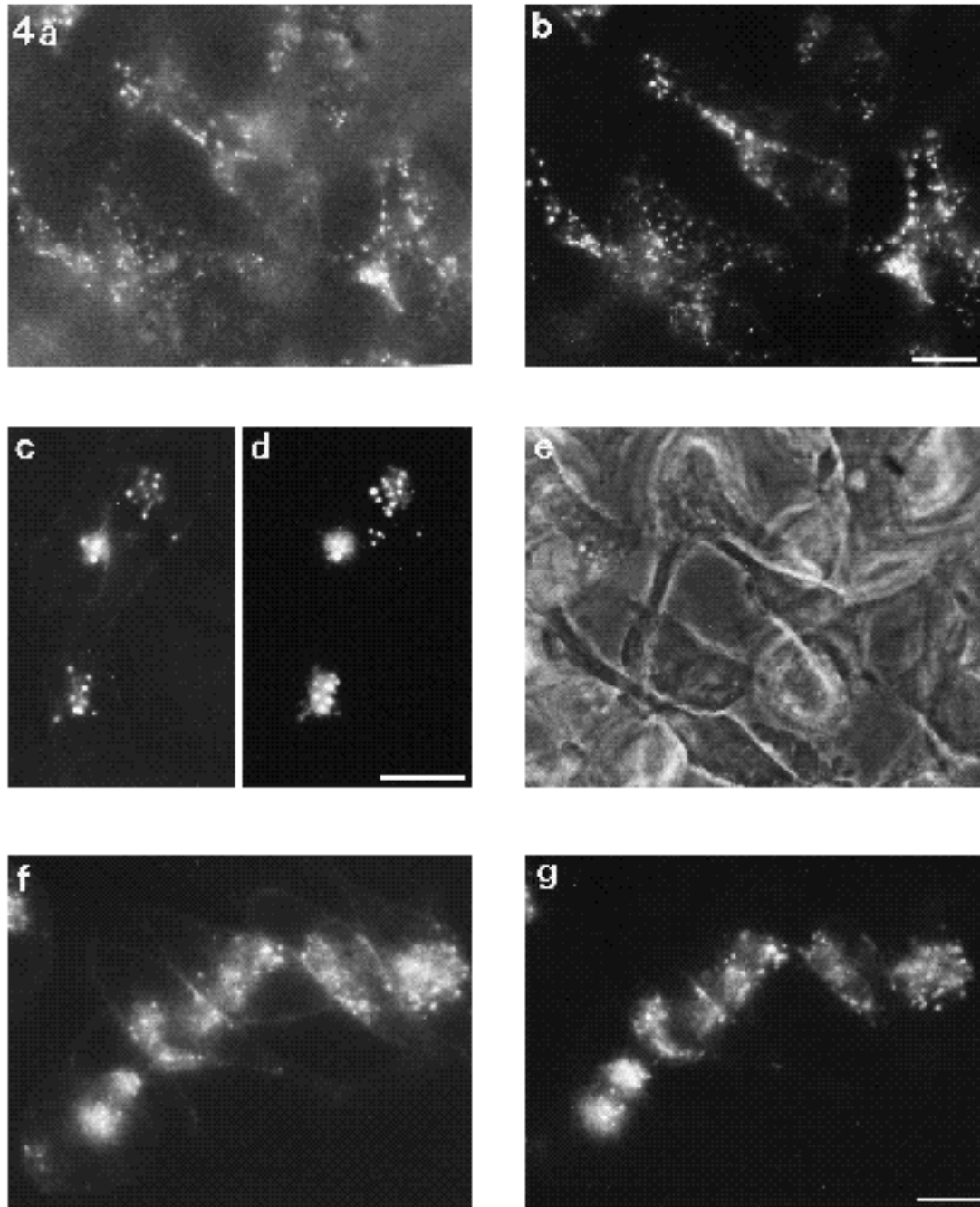
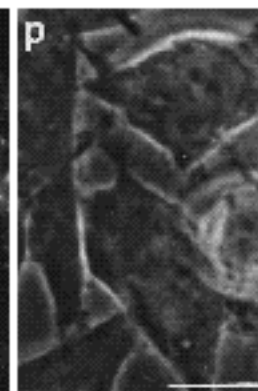
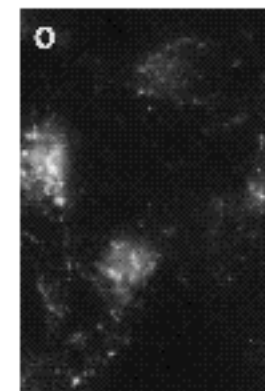
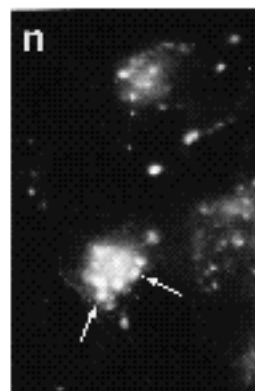
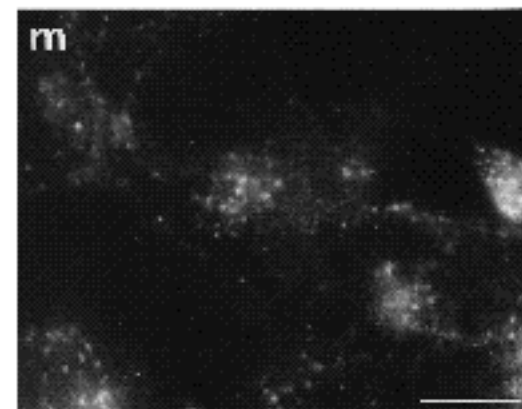
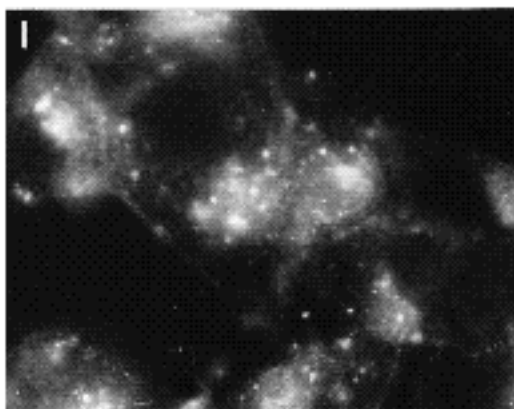
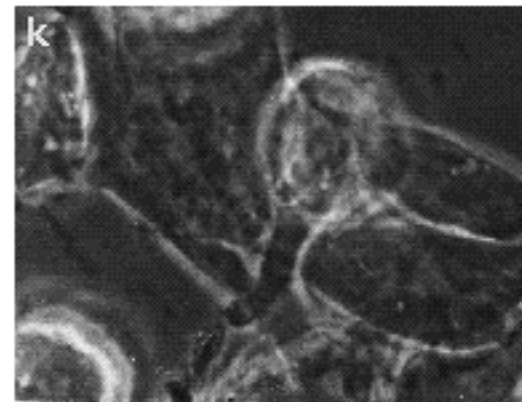
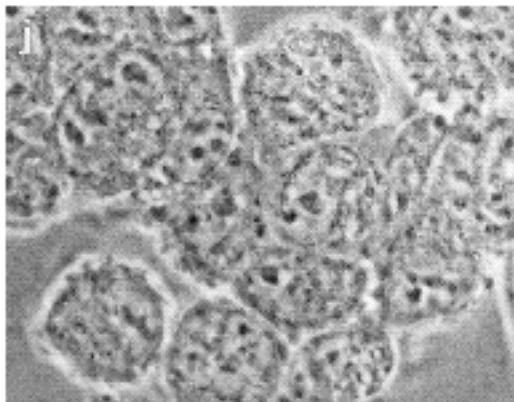
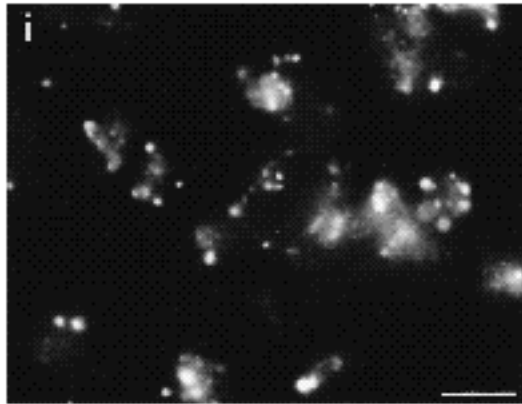
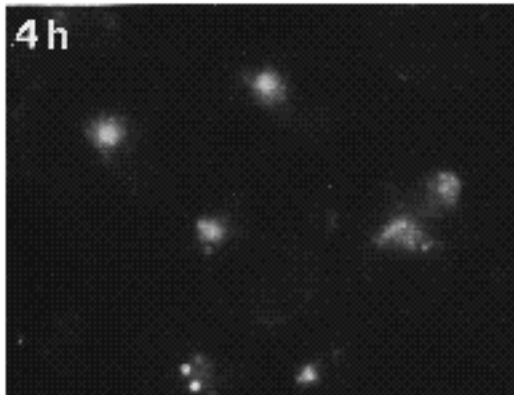


Fig. 4. Double-labeling patterns after internalization of C₆-NBD-glucosylceramide together with either LR-ricin or LR-Tf. (a-j) BHK cells were labeled with C₆-NBD-glucosylceramide (a,c,f,h) and LR-ricin (b,d,g,i), as described in Materials and methods. Subsequently the cells were incubated at 37°C for 2 min (a,b,e), 15 min (c,d), 30 min (f,g) and 60 min (h,i,j). Thereafter a back-exchange procedure and a 0.1 M lactose wash were performed. Cells shown in a,b,e were fixed, as described in Materials and methods. Up to 30 min (f,g) colocalization is observed, but after 60 min (h,i,j) very large spherical vesicles become labeled by ricin, but do not contain glucosylceramide. (k-p) BHK cells were labeled with C₆-NBD-glucosylceramide (l,n) and LR-Tf (m,o), as described in Materials and methods. Subsequently the cells were incubated at 37°C for 15 min, followed by a back-exchange procedure and fixing of the cells as described in Materials and methods. Phase-contrast image (k) corresponds to (l) and (m), whereas (p) corresponds to (n) and (o). Arrows indicate large spherical vesicles (late endosomes) which contain C₆-NBD-glucosylceramide, but not LR-Tf. Thus glucosylceramide colocalizes with ricin (c,d), but not with transferrin (k-p) in late endosomes after 15 min at 37°C. Bars, 10 μm.

Mechanism of recycling

Recycling does not depend on the presence of intact microtubuli or actin filaments. When cells were treated with noco-

dazole or cytochalasin D to disrupt these respective cytoskeletal elements, recycling was not inhibited (Figs 3 and 8).



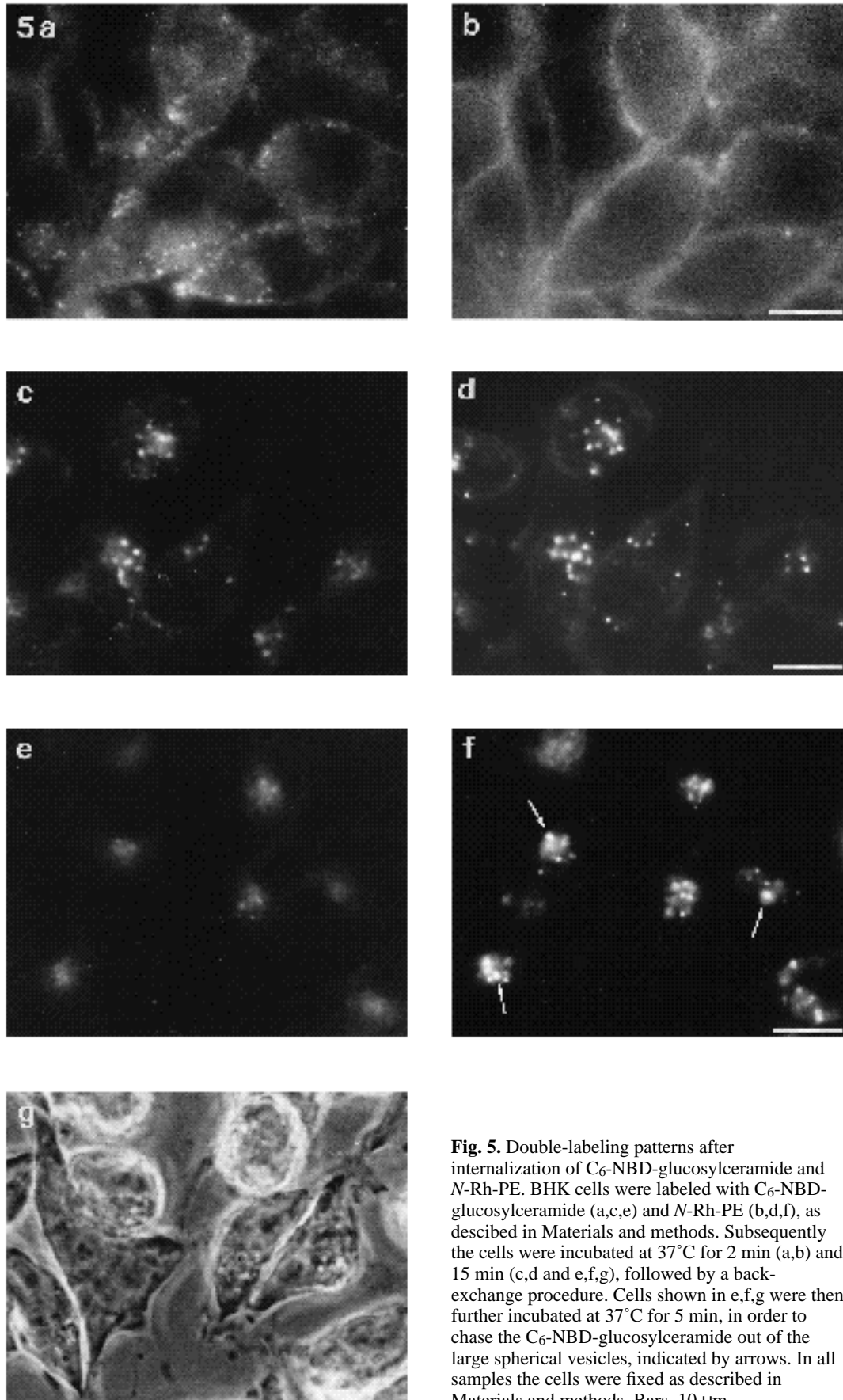


Fig. 5. Double-labeling patterns after internalization of C₆-NBD-glucosylceramide and N-Rh-PE. BHK cells were labeled with C₆-NBD-glucosylceramide (a,c,e) and N-Rh-PE (b,d,f), as described in Materials and methods. Subsequently the cells were incubated at 37°C for 2 min (a,b) and 15 min (c,d and e,f,g), followed by a back-exchange procedure. Cells shown in e,f,g were then further incubated at 37°C for 5 min, in order to chase the C₆-NBD-glucosylceramide out of the large spherical vesicles, indicated by arrows. In all samples the cells were fixed as described in Materials and methods. Bars, 10 μm.

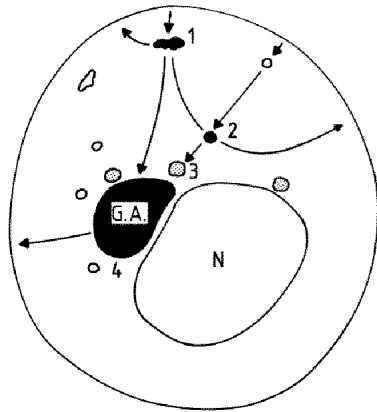


Fig. 6. A schematic view of the internalization and recycling pathways of C₆-NBD glucosylceramide in BHK cells. Glucosylceramide enters the cell along the pathway of receptor-mediated endocytosis, together with transferrin and ricin, and reaches the early endosomes (1). From this sorting compartment the glycolipid can - like transferrin - directly recycle to the plasma membrane. Apart from recycling, the glycolipid moves to late endosomes (2) and is thus partly sorted from transferrin, which is only transported to the Golgi area (G.A.) when moving further into the cell (4). Unlike transferrin, the other endocytic marker ricin does move to the late endosomes, together with glucosylceramide. In this late endosomal compartment a "cross-over" occurs between the receptor-mediated and fluid-phase endocytic pathways, since glucosylceramide and N-Rh-PE colocalize in this compartment (2) and not in early endosomes (1). Apparently, glucosylceramide can escape from this late endosomal compartment and thus avoid degradation in the lysosomal compartment (3), to which both N-Rh-PE and ricin are directed. In this way the glycolipid can recycle to the plasma membrane, either directly or via the Golgi area. However, the Golgi apparatus per se is not involved in this recycling pathway. During its intracellular movement in the endocytic pathway, a small fraction of the glucosylceramide is degraded to ceramide, which moves to the Golgi apparatus, where it can be reutilized for the synthesis of sphingolipids. This latter pathway is not depicted here. N, nucleus.

To characterize recycling further, several parameters were examined to determine whether or not recycling was affected. Ammonium chloride (20 mM), and both low (pH 5.5) and high (pH 9.5) *extracellular* pH did not influence recycling as judged by microscopy (not shown). However, when the *intracellular* pH was lowered to 5.5 using nigericin, recycling was severely inhibited (Fig. 8), while the intracellular distribution of fluorescence changed. Whereas in control cells the NBD-glycolipid is found in the cell center, in acidified cells the lipid analog is seen in irregularly shaped vesicles, scattered all over the cell (Fig. 8). Thus it appears that the extent to which the intracellular pH is lowered is of importance for the inhibitory effect on recycling, since simply lowering the extracellular pH is not sufficient. The latter method has been used to study pH-dependent internalization in BHK cells (Davoust et al. 1987). It was shown that by lowering the extracellular pH to 5.7 the intracellular pH dropped to 6.2. Apparently the threshold intracellular pH for inhibition of recycling is between 5.5 and 6.2. The pH-dependence suggests that recycling may

Table 2. Effect of metabolic inhibitors or acidification of the cytosol on C₆-NBD-glucosylceramide recycling

	Relative transport to plasma membrane (<i>R</i>)
Control	1
Metabolic inhibitors	0.59
Acidification cytosol	0.41

BHK cells were labeled with 4 μ M C₆-NBD-glucosylceramide and allowed to internalize the lipid for 30 min at 37°C, after which a back-exchange procedure was carried out to remove all residual plasma membrane NBD-lipid. Subsequently the cells were preincubated with Hanks' solution (control) or the same solution containing 5 mM sodium azide/50 mM 2-deoxyglucose (metabolic inhibitors), or a nigericin (5 μ M)-containing isotonic KCl buffer solution (acidification cytosol) for 30 min at 2°C, followed by incubation at 37°C for 45 min. Thereafter another back-exchange procedure was performed to analyse the NBD-lipid present in this (recycling) fraction and the residual intracellular fraction, as described in Materials and methods. Recycling is expressed as the relative transport factor *R*, as described in Materials and methods. Data are the means of triplicate measurements with standard deviations <10%. Note that Fig. 8 shows corresponding images of the NBD-glucosylceramide fluorescence distribution.

be accomplished by vesicular transport, in spite of the lack of microscopic visualization of carrier vesicles. It would be expected that the use of metabolic inhibitors would also affect recycling to a major extent, since such treatments have been reported to inhibit vesicular transport completely during internalization (Sleight and Pagano, 1984). In the presence of such inhibitors (5 mM sodium azide/50 mM 2-deoxyglucose) recycling was indeed inhibited, as judged by microscopy (Fig. 8). In this case intracellular C₆-NBD-glucosylceramide was still present in the cell center, as observed in control cells. As shown in Table 2, none of the inhibitors of recycling blocked the process completely. However, the extent of inhibition found in these experiments may be an underestimation, since in control cells part of the recycled C₆-NBD-glucosylceramide was reinternalized in the time-span of 45 min, whereas in acidified cells (re)internalization is effectively inhibited (Cosson et al. 1989; and our own unpublished observations). Furthermore, in contrast to acidification of the cytosol, which is a relatively fast process, the lag time before metabolic inhibitors exert their effect may be longer. This could explain the slightly less efficient inhibition of recycling under these conditions.

In conclusion, the present work demonstrates that recycling of a fluorescent analog of glucosylceramide can occur at the level of *early endosomes*, very similar to reports from a variety of proteins, such as Tf and its receptor. Also, the late endosomal compartment appears to play a role in glucosylceramide recycling. Involvement of early Golgi compartments seems to be very minor, but in all likelihood the TGN does play a role in recycling of the glycolipid. Defining this role more accurately will require further work. Whether the latter pathways reflect an 'overflow-salvage' mechanism, as has been suggested (see Hoekstra et al. 1989, and references therein) for Tf recycling via early endosomes and the Golgi complex also remains to be established.

Finally, the trafficking of glucosylceramide seems to resemble to a certain extent that of sphingomyelin in cultured fibroblasts (Koval and Pagano, 1989), thus indicating

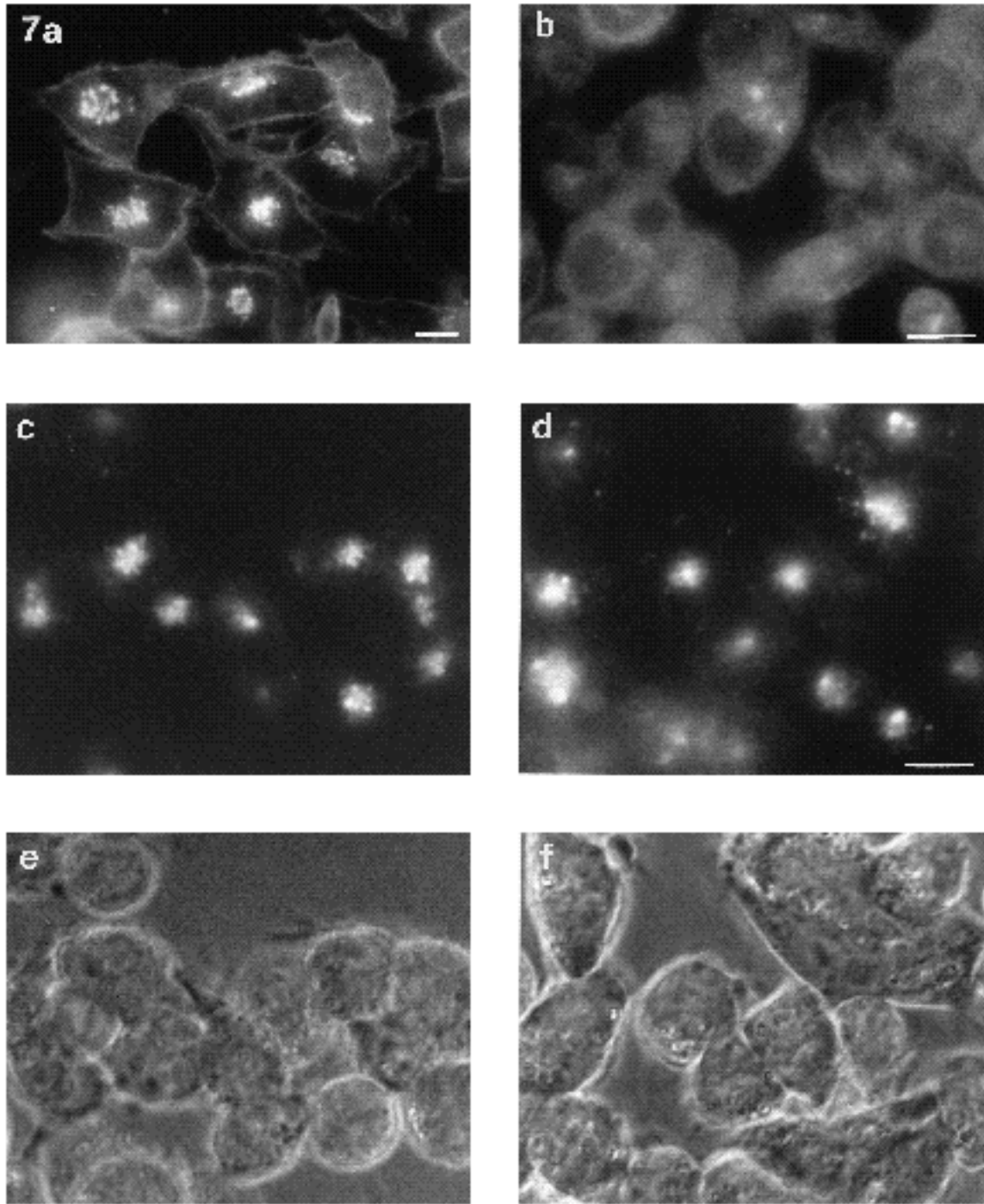


Fig. 7. Effect of BFA on the Golgi apparatus and the endocytic trafficking of C₆-NBD-glucosylceramide. (a) BHK cells were labeled with 4 μ M C₆-NBD-ceramide for 30 min at 37°C. (b) Cells were labeled as in a, followed by another 30 min incubation at 37°C in the presence of BFA (2 μ g/ml) and a back-exchange. Note that BFA treatment causes spreading of C₆-NBD-lipids from the Golgi all over the cell. (d) BHK cells were preincubated for 30 min at 37°C with BFA. Thereafter the cells were labeled with 4 μ M C₆-NBD-glucosylceramide (+ BFA) at 2°C, followed by a 30 min incubation at 37°C in the presence of BFA. Finally a back-exchange procedure was performed (f, corresponding phase-contrast image). (c) Control cells were treated as in d, but Hanks' buffer without BFA was used for all incubations (e, corresponding phase-contrast image). Note that BFA treatment does not change the NBD-glucosylceramide labeling pattern, under conditions where Golgi-localized NBD-lipids are spread all over the cell (b). Further note in Table 1 that BFA does not affect recycling of glucosylceramide, starting from the situation as in d. Bars, 10 μ m.

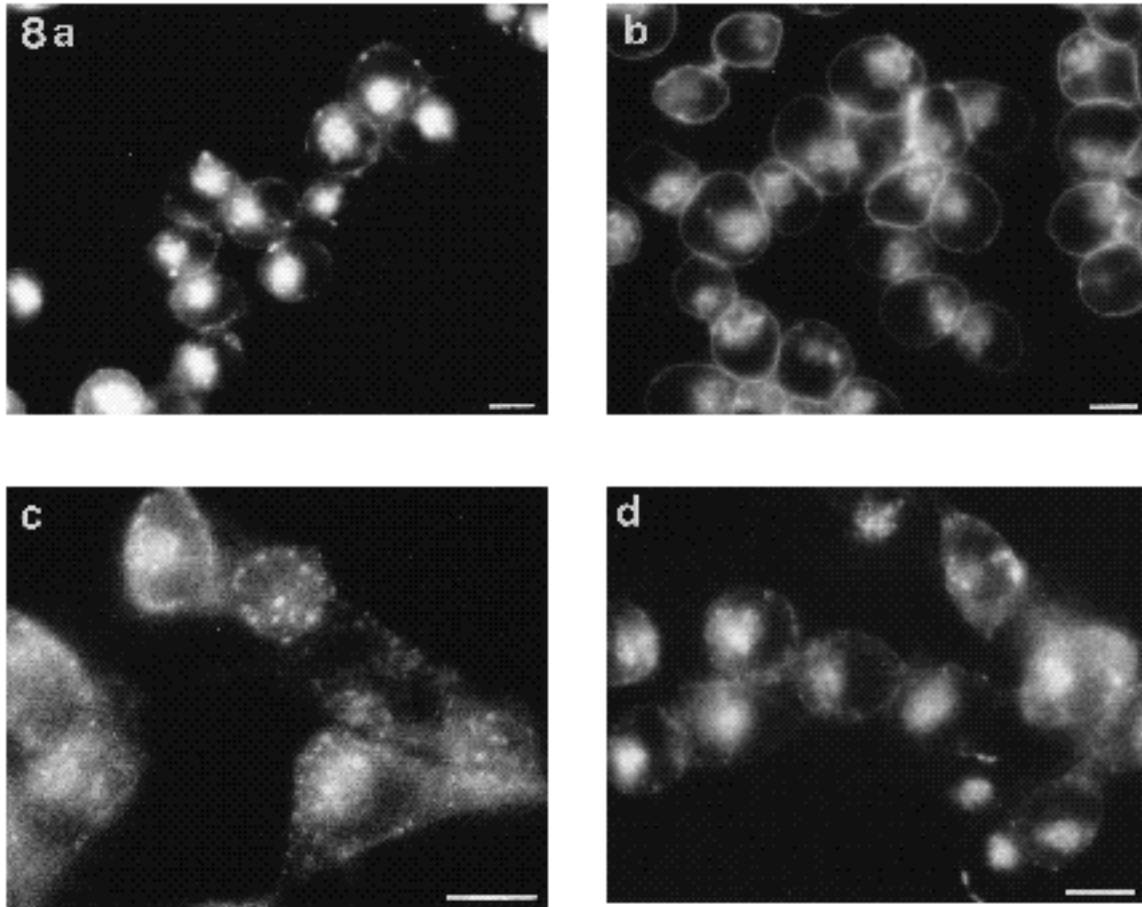


Fig. 8. Labeling pattern and recycling of C₆-NBD-glucosylceramide in BHK cells, treated with cytochalasin D or metabolic inhibitors, or after acidification of the cytosol. Cells were pretreated as indicated, labeled with 4 μ M C₆-NBD-glucosylceramide and allowed to internalize the lipid for 30 min at 37°C, followed by a back-exchange procedure. (a) Before labeling and monitoring internalization, the cells were preincubated for 30 min at 37°C with 10 μ g/ml cytochalasin D in Hanks' solution. The drug was present during all incubations. Note that trafficking of glucosylceramide to centrally located late endosomes is not inhibited by cytochalasin D. (b) Cells were treated as in a; after internalization and back-exchange, the cells were further incubated for 45 min at 37°C (in the presence of cytochalasin D). Plasma membrane staining indicates that recycling has occurred. (c) After internalization and back-exchange the cells were further incubated for 45 min at 37°C in nigericin-containing isotonic KCl buffer solution to acidify the cytosol. Note the peripheral location of C₆-NBD-glucosylceramide-containing vesicles in acidified cells. (d) After internalization and back-exchange the cells were preincubated for 30 min at 2°C in the presence of 5 mM sodium azide/50 mM 2-deoxyglucose, followed by a 45 min incubation at 37°C in the presence of these drugs. In contrast to b, in c and d no plasma membrane staining is observed, indicating that recycling of glucosylceramide is inhibited. For quantification of this inhibition see Table 2. Bars, 10 μ m.

that sphingolipids may follow similar routes in fibroblasts. However, the routing of (glyco)sphingolipids cannot be generalized for all cell types, since we have shown elsewhere that in the human (epithelial) carcinoma HT29 cell line, glucosylceramide is directed to the Golgi apparatus during internalization (Kok et al. 1991). Thus glucosylceramide follows different pathways in HT29 cells versus fibroblasts. These distinct differences in different cell types may provide interesting possibilities for examining the underlying mechanisms that direct glycolipid trafficking in cells.

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